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Rapid Microplate Assay for Substrates and Inhibitors of Proteinase Mixtures

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Evaluation of the hydrolysis of colorimetric substrates by impure proteinase mixtures has in the past been time-consuming and tedious, with the added economic disadvantage of using relatively large amounts of consumables, such as substrates and cuvettes. In the process of characterizing and purifying insect gut proteinases, we have used a microplate reader and a kinetic software program to incorporate efficiency and economy into procedures for rapidly identifying substrates and inhibitors useful in the analysis of proteinases.

We routinely use enzyme substrates conjugated to ρ-nitroanilide (pNA) to measure proteinase activity. Microplate screening of enzyme activity with substrates conjugated to pNA has been described for porcine pancreatic enzymes (2), but no kinetic data were given. We present a method for an enzyme microplate assay and use the procedure to compare substrate hydrolyses by several mammalian proteinases. In addition, we show how the effects of potential inhibitors on proteinase activity can be measured rapidly and easily, generating inhibitor curves and IC_{50} values. This technique will allow researchers to quickly identify inhibitors suitable for use in the isolation and characterization of proteins from crude extracts.

In the substrate assay, different pNA substrates (Sigma Chemical, St. Louis, MO, USA) were added at a concentration of 5 mg/mL in 100 µL assay buffer (0.1 M Tris-HCl, pH 8.1, 0.02 M $CaCl_2$) to individual wells in the top row (row A) of a 96-well microplate. All of the remaining wells (rows B–H) contained 50 µL of assay buffer. A multichannel pipettor was used to serially dilute (1:1) the substrates from rows A to G. Row H received no substrate, serving as a negative control. Enzymes (Sigma Chemical) were diluted in assay buffer, and 50 µL were added to each well with a multichannel pipettor. The amounts of enzymes added to each well were 10 µg of trypsin (bovine pancreatic, Type XI, DPCC-treated) or α-chymotrypsin (bovine pancreatic, Type II), or 1.77 U of elastase (porcine pancreatic, Type I). Samples were immediately incubated at 37°C in a microplate reader (Bio-Tek Instruments, Winooski, VT, USA) for 30 s to allow for temperature equilibration. Absorbance was then measured at 405 nm and monitored at 15-s intervals for 5 min. The mean velocities, in units of m*A*/min, were calculated by the software program KC3® (Bio-Tek Instruments).

Each mammalian enzyme was analyzed both individually and in combination for the ability to hydrolyze a variety of pNA substrates. From one microplate analysis, we obtained kinetic characterizations of three different pNA substrate hydrolyses by three mammalian enzymes and their mixtures. A representative graph of the data obtained from the hydrolysis of one substrate, *N*-succinyl-ala-ala-pro-phe ρ-nitroanilide (SAAPFpNA), which is diagnostic for chymotrypsin-like enzymes, is presented in Figure 1. The raw data from the kinetic software program were provided in units of m*A*/min, which were plotted against increasing substrate concentration. Approximately 90 min were required to set up the assay, measure the absorbances of the progress curves and perform graphical transformation of the data.

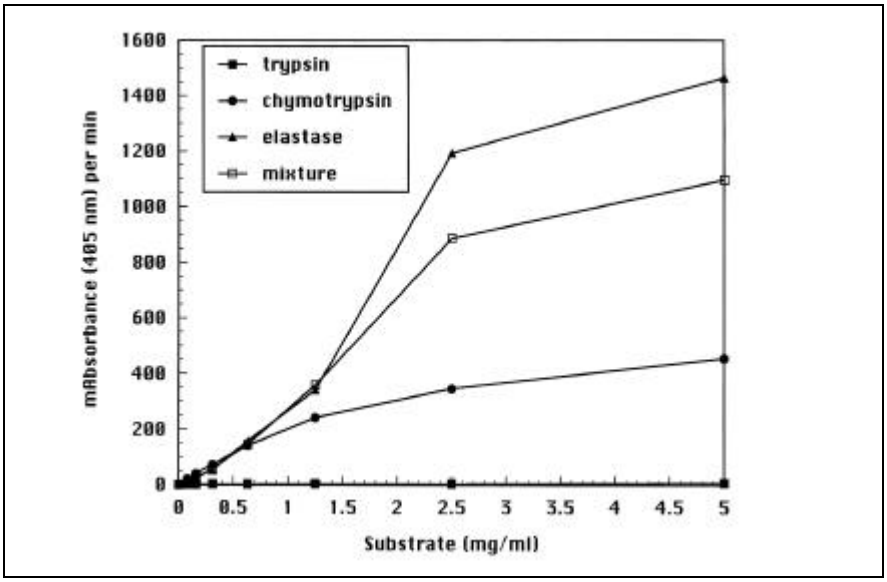

Figure 1. Data obtained from a proteinase substrate microplate assay. Rates of hydrolysis of SAAPFpNA by trypsin, chymotrypsin, elastase or a mixture thereof are given as a function of substrate concentration.

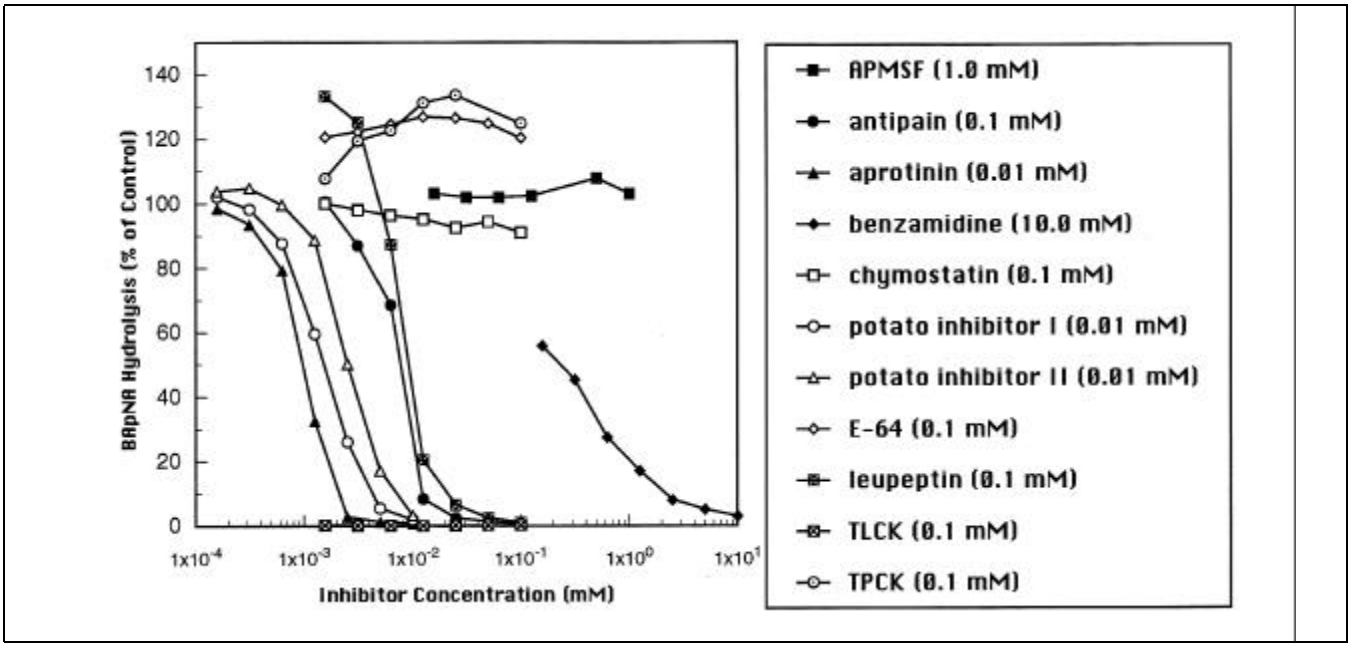

Figure 2. Data obtained from a proteinase inhibitor microplate assay. Relative hydrolysis of BApNA by trypsin in the presence of various inhibitors is given as a function of the concentration of inhibitor (mM). Activity was calculated from kinetic data as the percentage of activity obtained when no inhibitor was present. Initial concentrations of inhibitors before dilution are given in parentheses in the legend.

The procedure for the microplate analysis of inhibitor effects on proteinases was modified from the substrate analysis procedure. Inhibitors (from Sigma Chemical and Calbiochem-Novabiochem, La Jolla, CA, USA) were dissolved in 100 µL of assay buffer and added individually to the top wells (row A) of a 96-well microplate at the concentrations indicated in the legend of Figure 2. Serial dilutions of 1:1 were made using a multichannel pipettor in 50 µL of assay buffer from rows A to G. Fifty microliters of trypsin were added at a concentration of 10 µg per well, and the plates were preincubated at 37°C for 15 min before addition of substrate. To initiate the reaction, 50 µL of a 1-mg/mL solution of a trypsin-specific substrate, *N*-α-benzoyl-L-arginine ρ-nitroanilide (BApNA), were added to each well. The absorbance was monitored as in the substrate analysis procedure. Values obtained with the kinetic software program were converted using a transformation function in the program to percentage of control (no inhibitor). IC_{50} values were estimated using linear regression analysis of the enzyme activity vs. the natural logarithm of the concentration of the inhibitor.

The data obtained from a single microplate analysis of different inhibitors of trypsin are shown in Figure 2. The most effective inhibitor was TLCK, with essentially 100% inhibition observed over the concentration range tested (10^{-3}–10^{-1} mM). Aprotinin and potato inhibitors I and II were also effective in preventing trypsin hydrolysis of BApNA, with IC_{50} values of 1.02, 1.46 and 2.33 µM, respectively. Antipain and leupeptin were slightly less effective in trypsin inhibition, and benzamidine was inhibitory only at high concentrations (mM). Chymostatin, APMSF, E-64 and TPCK did not inhibit trypsin hydrolysis of BApNA at the concentrations tested. All inhibitors predicted to inhibit trypsin did so with the exception of APMSF. We attribute the lack of inhibition of trypsin by APMSF to the instability of the inhibitor at the pH of the buffer used in the assay. APMSF has a half-life of only 1 ms at pH 8.0 (1). Using a single microplate, eleven inhibitors were screened over the dose range, and the entire procedure required only about 2 h to complete.

This microplate technique was developed to analyze complex mixtures of proteinases. Substrates containing pNA-amino acid conjugates can be screened easily and quickly by this technique. In this assay, values obtained from a mixture of mammalian enzymes, each specific for a particular substrate, were similar to values obtained using individual enzymes. Inhibition curves were generated using serial dilutions of a variety of inhibitors in a single microplate. We have successfully used this method to partially characterize proteinases from *Plodia interpunctella*, an insect pest of stored products, and obtained data for the substrate specificity and inhibitor susceptibility of the major enzymes in gut extracts (3). The procedure can be used to study any enzyme that hydrolyzes a substrate that can be monitored spectrophotometrically.

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Isolation of Site-Specific Insert Probes from Chimeric YACs

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The yeast artificial chromosome (YAC) cloning system (3) and the development of "Mega YACs" with inserts greater than 1 Mb (1,4) have proven to be a powerful approach in the analysis of complex genomic regions (5). However, detailed analysis of YAC libraries showed that a significant proportion of clones contains two or more noncontiguous pieces of DNA (6). These so-called chimeric YACs, obtained either from somatic cell hybrid DNA or from total human DNA, can cause substantial problems with methods in genome research or with diagnostic applications in particular techniques such as cDNA selection or mapping of insert DNA by fluorescence in situ hybridization (FISH) analysis.

The method we describe offers a convenient and reliable approach to separate the YAC insert DNA from yeast DNA and to isolate DNA fragments from different genomic regions of chimeric YACs. It is based on the occurrence of two CpG dinucleotides in the restriction site of the rare cutting restriction enzyme *Mlu*I. *Hpa*II tiny fraction (HTF) islands, which contain a high density of nonmethylated CpG islands, are clustered at the 5′ ends of human genes, where they are involved in the control of the transcription process (2). This is in contrast with the yeast genome, where CpGs are randomly distributed.